

AD _____

Award Number: DAMD17-98-1-8172

TITLE: Novel Vector System for Breast Cancer Therapy

PRINCIPAL INVESTIGATOR: Robert I. Garver, Ph.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, Alabama 35294-0111

REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

DTIC QUALITY INSPECTED 4
20010122 129

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE October 1999	3. REPORT TYPE AND DATES COVERED Annual (1-Oct-98 - 30-Sep-99)
4. TITLE AND SUBTITLE Novel Vector System for Breast Cancer Therapy		5. FUNDING NUMBERS DAMD17-98-1-8172
6. AUTHOR(S) Robert I. Garver, Ph.D.		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Alabama at Birmingham Birmingham, Alabama 35294-0111		8. PERFORMING ORGANIZATION REPORT NUMBER
E-MAIL: robgarver@sprintmail.com		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES		
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited		12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Experiments outlined in the original statement of work showed that our initial sustained release technology for the delivery of therapeutic adenovirus was not feasible. As a result, we have developed a revised statement of work that entails the development of a controlled release formulation of tumor necrosis factor α (TNF α) in combination with an adenovirus that replicates selectively within carcinoma tissue and sensitizes the tumor tissue to TNF α . We have succeeded in developing microspheres that release bioactive TNF α over more than 1 month. We have also succeeded in obtaining the selectively replicating adenovirus (d338). We have also succeeded in demonstrating a greater than additive tumoricidal activity <i>in vitro</i> of the TNF α combined with the d338. These experimental successes will be followed in the current year by animal experiments expected to validate this novel therapy for breast carcinoma.		
14. SUBJECT TERMS Breast Cancer		15. NUMBER OF PAGES 10
		16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified
		20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

DL Where copyrighted material is quoted, permission has been obtained to use such material.

DL Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

DL Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

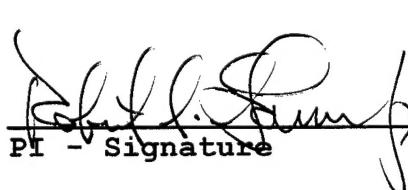
In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.


Robert L. Smith
PI - Signature 11/4/99
Date

4. TABLE OF CONTENTS

1.	Front Cover.....	1
2.	Standard Form (SF) 298.....	2
3.	Foreword.....	3
4.	Table of Contents.....	4
5.	Introduction.....	5
6.	Body.....	5-8
7.	Key Research Accomplishments.....	8
8.	Reportable Outcomes.....	8
9.	Conclusions.....	8
10.	References.....	9

Progress Report for DAMD17-98-1-8172

5. INTRODUCTION

The original grant proposal was directed towards the development of targetable microspheres that would release recombinant adenovirus over a sustained period of time within breast carcinoma tissue. This project is a collaborative effort between the Department of Biomedical Engineering at Johns Hopkins University (JHU) and the Department of Medicine at the University of Alabama at Birmingham (UAB). At the time of the grant proposal submission, we had developed a novel microsphere formulation that released adenovirus in a time-dependent fashion [1]. An important aspect of this original formulation was the finding that the microspheres could be lyophilized, thereby stabilizing the formulation. In subsequent experiments performed by both UAB and JHU, we found that it was difficult to reproduce the successful retention of adenoviral bioactivity following lyophilization. The reasons for this will be discussed in the subsequent sections, however, we felt this was a major impediment to the completion of our original aims.

We have redirected our efforts towards a different controlled release system that is used in conjunction with a therapeutic adenovirus, and this is reflected in a modified Statement of Work (attached). The new direction involves the development of a controlled release tumor necrosis factor alpha (TNF α) that will be used in combination with a conditionally replicative adenovirus containing an intact E1A transcriptional unit. The rationale for this new direction is based on several points as follows: (i) TNF α is toxic when administered systemically so that local, controlled release will increase the therapeutic index, (ii) cells expressing the adenovirus E1A proteins have been shown by multiple investigators to be sensitized to the toxic effects of TNF [2-7], hence using an E1A adenovirus with TNF should result in a combined toxicity, (iii) the E1A-containing adenovirus is similar to a virus shown to have a biological selectivity for replication in neoplastic tissues [8-11], even when systemically administered - thereby providing an element of biological targeting of breast carcinoma tissues.

6. BODY

Original statement of work, task #1:

a) microspheres made with varied percentages of gelatin, alginate and calcium - other variables examined included systematic changes in the temperature of gelatin, alginate - we also carefully examined influence of vortex speed on size and size variability of the spheres:

Result summary - Although we did identify conditions that resulted in consistently sized spheres, we found that lyophilization reduced bioactivity by 2-3 orders of magnitude. We subsequently tried different lyophilization buffers (varying glycerol concentration) without any improvement. We consulted several biotechnology companies, learning that the only means of consistently preserving adenoviral bioactivity with lyophilization required a proprietary process with expensive, gradual lyophilization equipment used in the pharmaceutical industry. This technical barrier was felt to be insurmountable within the budgetary constraints of this grant, hence we shifted

efforts into our new statement of work.

Revised statement of work, task #1:

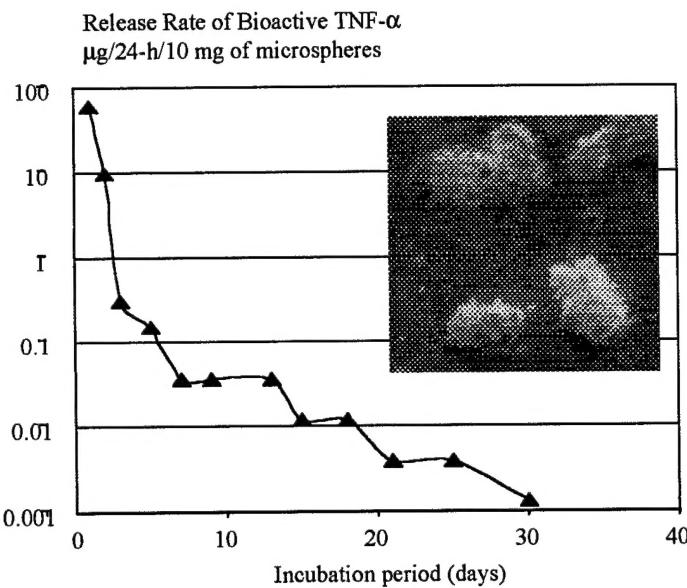
Synthesis of TNF-a Controlled Release Microspheres

Human serum albumin (HSA, 2.5%) was prepared from the injectable 25% HSA solution (Albumarc 25%, Baxter Healthcare Co., CA) and adjusted to pH 3.0. Heparin (1000 USP, Elkins-Sinn, NJ) purchased from the hospital pharmacy was used without any modification. TNF- (10^5 U/ml) was added to the heparin solution before the coacervation. Microsphere formulation was achieved by adding HSA solution (3 ml) into a vortexing heparin solution (3 ml). After 10 sec of vortexing, the crosslinking reagent 1-ethyl 3 (3-propylamino) carbodiimide hydrochloride (EDC) was added to a final concentration of 3 mg/ml. After 15 min of reaction at RT, 0.1 M glycine (7ml) was added, and kept for another 15 min to quench the unreactive EDC. The typical encapsulation efficiency of TNF- was close to 95%.

In vitro Release studies

In vitro release studies were conducted by incubating the microspheres in 10% FCS medium at 37°C. The bioactivity of the released TNF- was assessed by determining the cytotoxicity of the cytokine on HGC-27 cells. Briefly, HGC-27 cells were seeded at a concentration of 5×10^4 cells/well in 100 l culture medium containing actinomycin C₁ (1g/ml) and 100 l of the released medium into microtiter plates. After incubation for 24 h at 37°C and 5% CO₂, 10 l of cell proliferation reagent WST-1 was added to the wells and incubated for an additional 4 h. The absorbance of the wells ($A_{450\text{nm}} - A_{690\text{nm}}$) was measured and compared to a calibration curve to determine the bioactive concentration of TNF-.

The TNF- microparticles are irregular in shape, with a particle size range of 5-20 m. Release of TNF- from the microparticles follows a first-order kinetics and there is a burst in the first 24 h, although bioactive cytokine can still be detected for up to 3 weeks.

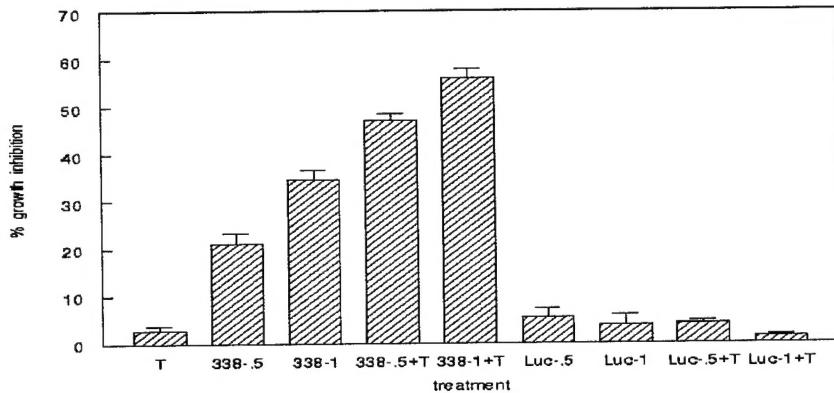


Task #2: Identify a conditionally replicative adenovirus suitable for use with the TNF α formulation

The objective here was the acquisition of an adenovirus that would efficiently transfer the E1A proteins into breast carcinoma tissue in order to sensitize the tissue to TNF α . The optimal virus would replicate selectively within the carcinoma tissue, and not in surrounding normal tissue so as to enhance the transfer of the adenoviral E1A within the tumor tissue. Using other funding sources, we had developed a virus (AdE1A-tk) that we had hoped would fulfill this objective. However, in pilot animal studies, we found that the virus did not replicate well within tumor nodules. As an alternative, we obtained the *dl338* adenovirus for use in these studies. This virus contains a deletion within the E1B 55 kD -encoding region that greatly reduces its replication within normal tissues [12]. This virus is biologically similar to the Onyx-015 virus that has been developed as a tumor-specific oncolytic agent, and is now in Phase II clinical trials. We obtained a stock of this virus, and amplified it by standard methods. The identity of the virus was confirmed by selective sequencing of the E1B region.

Task #3: Evaluate individual and combined activity of the *dl338* virus and TNF α *in vitro*

The *dl338* virus and TNF α were used individually or in combination in the treatment of the lung carcinoma cell line, A549, *in vitro*. The results shown here are the mean of 3 experiments, and clearly demonstrated that the combination of virus and TNF α caused a significantly greater reduction in carcinoma cell growth than either of the treatments individually. For example, the TNF alone caused a 4% reduction, *dl338* alone (moi=0.5) a 22% reduction, but the combination resulted in a 44% reduction.



Reduction of A549 growth by TNF α and/or adenovirus. Shown is the mean of 3 experiments \pm S.E.M. Ordinate: % growth reduction determined by a colorimetric growth assay 5 days after treatment, abscissa: agents administered to the cells. "T"=TNF α 100 ng/ml, "338"=d338 with moi following hyphen, "Luc"=AdLuc, a control adenovirus lacking E1A region.

7. KEY RESEARCH ACCOMPLISHMENTS

- development of controlled release formulation of TNF α as a novel cancer therapeutic
- development of a novel therapeutic strategy for the treatment of carcinoma that employs sustained release TNF and adenovirus with an intact E1A region - feasibility demonstrated by *in vitro* experiments

8. REPORTABLE OUTCOMES

- invention disclosure filed with university for use of sustained release formulation of TNF

9. CONCLUSIONS

The original research plan has been revised in response to the unexpected technical difficulties encountered that called into question the ability to complete the subsequent tasks. A revised Statement of Work has been developed that incorporates similar themes as in the original proposal. This new directed is also highly novel, and employs the first described sustained release formulation of TNF α in combination with a selectively replicating adenovirus with anticipated biological selectivity for breast carcinoma tissue. In the next twelve months, we plan to continue progress along the revised Statement of Work with completion of the initial animal experiments.

10. REFERENCES

1. Kalyanasundaram, S., Feinstein, S., Nicholson, J. P., Leong, K. W., and Garver, R. I., Jr., *Cancer Gene Ther.*, 6, 107-112 (1990).
2. Chen, M.-J., Holskin, B., Strickler, J., Gorniak, J., Clark, M. A., Johnson, P. J., Mitcho, M., and Shalloway, D., *Nature*, 330, 581-583 (1987).
3. Duerksen-Hughes, P. J., Hermiston, T. W., Wold, W. S. M., and Gooding, L. R., *J. Virol.*, 65, 1236-1244 (1991).
4. Hashimoto, S., Ishii, A., and Yonehara, S., *International Immunology*, 3, 343-351 (1991).
5. Ames, R. S., Holskin, B., Mitcho, M., Shalloway, D., and Chen, M.-J., *J. Virol.*, 64, 4115-4122 (1990).
6. Wold, W. S., *J. Cell. Biochem.*, 53, 329-335 (1993).
7. Tsuji, Y., Ninomiya-Tsuji, J., Torti, S. V., and Torti, F. M., *J. Immunol.*, 150, 1897-1907 (1993).
8. Bischoff, J. R., Kirn, D. H., Williams, A., Heise, C., Horn, S., Muna, M., Ng, L., and Nye, J. A., *Science*, 274, 373-376 (1996).
9. Kirn, D. H. and McCormick, F., *Molecular Medicine Today*, 2, 519-527 (1996).
10. Heise, C., Sampson-Johannes, A., Williams, A., McCormick, F., Von Hoff, D. D., and Kirn, D. H., *Nature Medicine*, 3, 639-645 (1997).
11. Kirn, D., Ganley, I., Nemunaitis, J., Otto, R., Soutar, D., Kuhn, J., Heise, C., Propst, M., Maack, C., Eckhardt, G., Kaye, S., and Von Hoff, D., *Cancer Gene Ther.*, 4, S13 (1997).
12. Pilder, S., Moore, M., Logan, J., and Shenk, T., *Mol. Cell. Biol.*, 6, 470-476 (1986).

DAMD17-98-1-8172

Statement of Work-Revised 10/99

1. Task #1: Construct and characterize microspheres that contain and release tumor necrosis factor α (TNF α) over an extended period of time
 - a) identify a formulation that encapsulates TNF α in a bioactive form
 - b) develop an assay that can measure small amounts of TNF α released from the encapsulated formulation
 - c) modify the above formulation to release TNF α over a 10-30 day time period
2. Task #2: Identify a conditionally replicative adenovirus suitable for use in combination with the extended release TNF α formulation
 - a) obtain *dl338* virus
 - b) amplify virus, confirm identity by limited sequence analysis of E1B region
3. Task #3: Evaluate individual and combined activity of *dl338* virus and TNF α *in vitro*
 - a) test *dl338* and TNF α on lung carcinoma cell line *in vitro*
 - b) test *dl338* and TNF α on MCF7 breast carcinoma cell line *in vitro*
4. Task #4: Evaluate the combined activity of *dl338* virus and TNF α *in vivo* by intratumoral injection
 - a) administer the *dl338* and TNF α by intratumoral injection to MCF7 tumor nodules
 - b) assess distribution of virus in MCF7 tumor nodule by rescue cultures and PCR analysis
5. Task #5: Evaluate combined activity of *dl338* virus administered systemically and TNF α administered by intratumoral injection
 - a) administer the *dl338* to tumor bearing mice by tail vein injection, and administer the TNF α formulation by intratumoral injection
 - b) assess distribution of virus in MCF7 tumor nodule by rescue cultures and PCR analysis